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Research Article

PARTIAL PURIFICATION OF PHENOL-DEGRADING ENZYME FROM *RHODOCOCCUS* UKMP-5M

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ABSTRACT
The feasibility of using *Rhodococcus* UKMP-5M in phenol biodegradation is the main interesting in this study. *Rhodococcus* species degrading phenol through enzymatic action of phenol hydroxylase. The molecular weight of phenol hydroxylase was successfully detected at 53 kDa. The presence of this enzymes have demonstrated that *Rhodococcus* UKMP-5M is a bacterium which has a great potential to be used industrially in the removal of xenobiotic compounds especially phenol.

Keywords: *Rhodococcus UKMP-5M, Biodegradation, Phenol, Protein*

INTRODUCTION
Phenol is a characteristic toxic pollutant in wastewaters of coal conversion processes, effluents from crude oil and treatment plants (Alamzadeh *et al.,* 2002). Many studies have been carried out by researchers for the improvement of phenol degradation process such as using various bacteria such as *Acinetobacter* sp. (Ahmad *et al*., 2011a), *Alcaligenes* sp. (Bastos *et al*., 2000). *Candida*sp. And *Rhodococcus* sp (Prieto *et al.,* 2002).
Study on phenol hydroxylase gene using *Rhodococcus* UKMP-5M by Norazah *et al*., (2012) noted the presence of 1500bp band that encoded for phenol hydroxylase enzyme for degradation of phenol. Purified phenol hydroxylase from *Rhodococcus* UPV-1 using sepharose fast-flow column shows a dimmer of 62 kD and 22 kDa (Saa *et al*., 2010). Meanwhile, phenol hydroxylase from *pseudomonas stutzeri* OX1 shows three molecular weights, which were 13, 38 and 60 kDa when purified using Q-sepharose fast flow, sephacryl S300 high resolution and superose 12 PC (Cafaro *et al*., 2004). *Candida tropicalis* purified using DEAE-sepharose, fractionation by polyethylene glycol 6000 and gel permeation chromatography of sepharose 4B shows a molecular mass of 240 kDa consisting of four identical subunits with a molecular mass of 60 kDa, indicating that this enzyme is a tetramer with 4.2 % yield (Paca Jr *et al*., 2007). *Bacillus thermoglucosidasius* purified using gel filtration show molecular weight of 120 and 35 KDa (Kirchner *et al*., 2003). The objective of this study was to determine the phenol hydroxylase molecular weight from phenol degrading bacteria, *Rhodococcus* UKMP-5M.

MATERIALS AND METHODS
Microorganism and Inoculum Preparation
The bacterium, *Rhodococcus* UKMP-5M, was used throughout this study. This bacterium was isolated from a petroleum contaminated soil at an oil refinery in Malacca, Malaysia. The bacterium from stock culture maintained in glycerol was transferred into shake flask containing nutrient broth (Merck, Germany). The flask was incubated at 30^{o}C in an incubator shaker (Jeio Tech SI-600R, Korea) agitated at 160 rpm for 24 h. This culture was used as a standard inoculum throughout this study.
Cultivation and Phenol Degradation Experiments
The Minimal Salt Medium (MSM) for batch fermentation of phenol degradation as proposed by Bai *et al* (2007) was used in this study. The optimized $(NH_4)_2SO_4$;0.3 g/L was used to obtain the highest concentration of cell. The medium (100 mL) in 250 mL shake flask was inoculated with 10%

(v/v) inoculum to initiate the cultivation and degradation of phenol. The flask was incubated at 36^{o}C on a rotary shaker, agitated at 160 rpm. During the cultivation, 10 mL of culture samples were withdrawn at time intervals for analysis.

Analytical Procedures

During the fermentation, 10 mL of samples was taken at different time intervals for chemical study. The sample was centrifuges at 40,000 rpm for 20 minutes. The cell pellet was used for the determination of enzymes activities.

Intracellular Enzyme Assay

Preparation of Cell Extraction

Sample was centrifuged at 40,000 rpm for 20 min at 4^{o}C. Cell pellet with ratio 1 g cell per 20 mL deionized water was mixed with acid-washed glass beads at the ratio of 1.5 g of glass beads per mL of medium (50 mM phosphate buffer at pH 7.5) containing 0.5 mM dithiothreitol as an anti protease in a 50 mL Falcon tube (Ramanan *et al*., 2008). This study was conducted in rotary shaker incubator at 25^{o}C for 30 min. The shaking speed was set at 275 rpm. The cell debris was removed by centrifugation at 40,000 rpm at 4^{o}C for 20 min. The supernatant (crude extract) was used for enzymes and protein assays.

During the cultivation, samples were taken for analysis. The samples were centrifuged at 40,000 rpm for 20 minutes. Phenol hydroxylase enzyme assay was based on method carried out by Ali *et al.*, (1998) with some modification. The phenol hydroxylase molar extinction coefficient at 340 nm is 6200 M-1 cm-1 (Dawson, 1985).

Protein Determination

Total protein content was measured using Bradford method (Bradford, 1976). 40 µL supernatant or crude was mixed with 1 mL Bradford reagent. Bradford reagent was previously diluted with ratio 1 deionized water to 4 bradford reagent (refer to Bio-Rad protein assay kit manual).

Bovine Serum Albumin (BSA) was used as a standard in the linear dilution range from 0.2 to 1.0 mg/mL. The absorbance of the resulting solution after incubation for 5 min (blue in colour) was measured at 595 nm using a spectrophotometer.

Total protein content was measured using Bradford method (Bradford, 1976).

Phenol Hydroxylase Enzyme Assay

The assay was based on method carried out by Ali *et al*., (1998) with some modification. The oxidation of NADH in the presence of phenol by physically-treated cell extracts was monitored at 340 nm for every 1.5 min. Three milliliters of reaction mixtures containing 50 mM KH_2PO_4: K_2HPO_4 buffer pH 7.2, 100 µmol NADH, and 100 µmol phenol was used before the addition of the 100 µL cell extract.

One unit of activity was defined as the amount of enzyme catalyzing the oxidation of 1 µmol NADH per minute. The phenol hydroxylase molar extinction coefficient at 340 nm is 6200 $M^{-1}cm^{-1}$ (Dawson, 1985) and the enzyme activity was calculated based on Beer's Law**.**

Purification of Phenol Hydroxylase Enzyme

Purification Using Ion Exchange Chromatography

Fully automated protein purification system Akta Explorer 100, based on liquid chromatography was used for Phenol Hydroxylase Enzyme purification using ion exchange medium. Software used was Unicorn 5.0 in the instrument. Purification of the phenol hydroxylase was carried out using ion exchange medium HITrap DEAE-Sepharose Fast Flow. Tris buffer used as a binding buffer and the same buffer was used to prepare crude samples.

The elution buffer used was Tris buffer with NaCl. Tris buffer with NaCl was also used to regenerate the medium. After regeneration, column was washed with Tris buffer, following the filtered ultra-pure water washing and kept in 20% alcohol.

Flow rate used was 1 mL/min with maximum backpressure of 0.3 Mpa and three bar. The fractions collected were analyzed for total protein and phenol hydroxylase enzyme. Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE) was carried out in this experiment. SDS-PAGE separations (Laemmli, 1970) were run in denaturing conditions.

Polyacrylamide Gel Electrophoresis

Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

To set up gel for SE 600 Ruby (GE Healthcare), running gel consisted of 10 mL monomer solution, 7.5 mL 4x running buffer (1.5M Tris-HCL, pH 8.8), 300 µL 10% SDS and 12.1 mL distilled water. The gel solution is vacuum for 15-20 min then 150 µL of 10% ammonium persulfate (APS) was added. APS must be preparing freshly. The 10 µL of Temed was added to the solution. The running gel solution is pour into Ruby unit and 1.5 mL of running gel overly (0.375M Tris-HCL, 0.1% SDS, pH8.8) is added above. The gel let to polymerize for 1 h and then pour out before adding stacking gel. Stacking gel contained 1.33 mL monomer, 2.5 mL stacking gel buffer and 100 µL 10% SDS. 6 mL distilled water was then added. The gel was vacuum for 15 min. Finally, 50 µL 10% APS and 5 µL Temed was added. Comb is put between the glasses and left for another 1 hour. Sample was added with 2 times treatment buffer (2x TB) and incubated at 100^{o}C in water bath stem for 90 second. For 10kD marker, 8 µL samples are required for loading. Sample was load before the current was set at 14 Amp for overnight. Commassie staining was used for staining procedure.

RESULTS AND DISCUSSION

Purification of Phenol Hydroxylase

Rhodococcus UKMP-5M has been known as a phenol degrading bacterium, which occurs through the action of the phenol hydroxylase enzyme via a meta-cleavage pathway. Figure 1 shows the SDS polyacrylamide gel indicating that the phenol hydroxylase was detected at 53 kD. This result is in agreement with the predicted molecular mass of phenol hydroxylase enzyme based on amino acid sequence of *Rhodococcus* UKMP-5M (Mokhtar *et al.,* 2008). Study on phenol hydroxylase gene using *Rhodococcus* UKMP-5M by Norazah *et al*., (2012) noted that the presence of a 1500bp band that encoded for phenol hydroxylase enzyme.

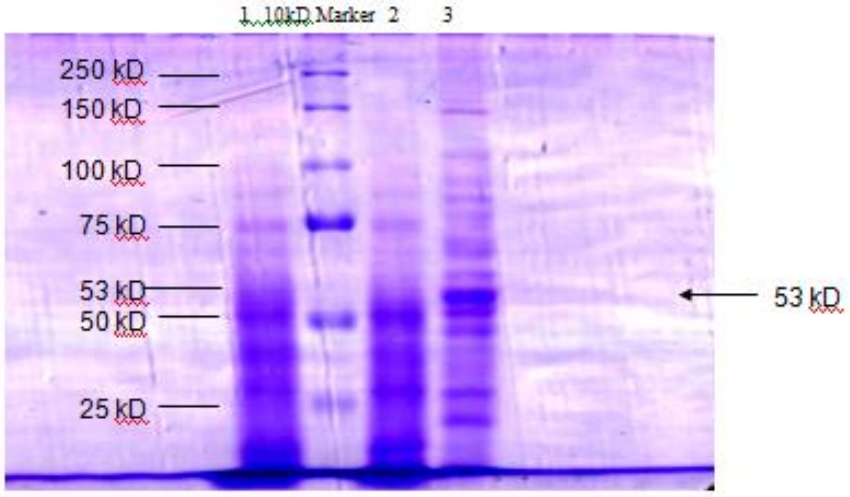

Figure 1: SDS Polyacrylamide Gel of the *Rhodococcus* UKMP-5M at Molecular Weight of 53 kD Phenol Hydroxylase; Lane 1 and 2, 10,000 MWCO Crude Extract and Lane 3, Unconcentrated Crude Extract; (From Culture of *Rhodococcus* UKMP-5M Cultivated in MSM with 0.78 g/L Phenol

The purification of phenol hydroxylase was carried out using the cell-free extract, where cells were previously disrupted using glass bead method. DEAE HiTRAP Fast Flow ion exchange column was used in enzyme purification. The purification procedure was started by running the cell-free crude extract through the DEAE-Sepharose fast flow. The elution profile is shown in Figure 2 (A). Phenol hydroxylase was detected in two peaks at 1M Nacl, which was tube 11 and 12. NaCl (1 M) was used to elute the

enzyme. Polyacrylamide gel electrophoresis (PAGE) was used to separate protein based on their size and molecular weights. Figure 2 (B) shows the SDS-PAGE results for phenol hydroxylase obtained after partial purification. The gel shows that the number of protein bands decreased after the purification step. Even though there are, more than one band appears on gel, the clear band was determined after the ion exchange step with a molecular weight of 53 kDa. This is identical to the molecular weight estimated from the amino acid protein sequence.

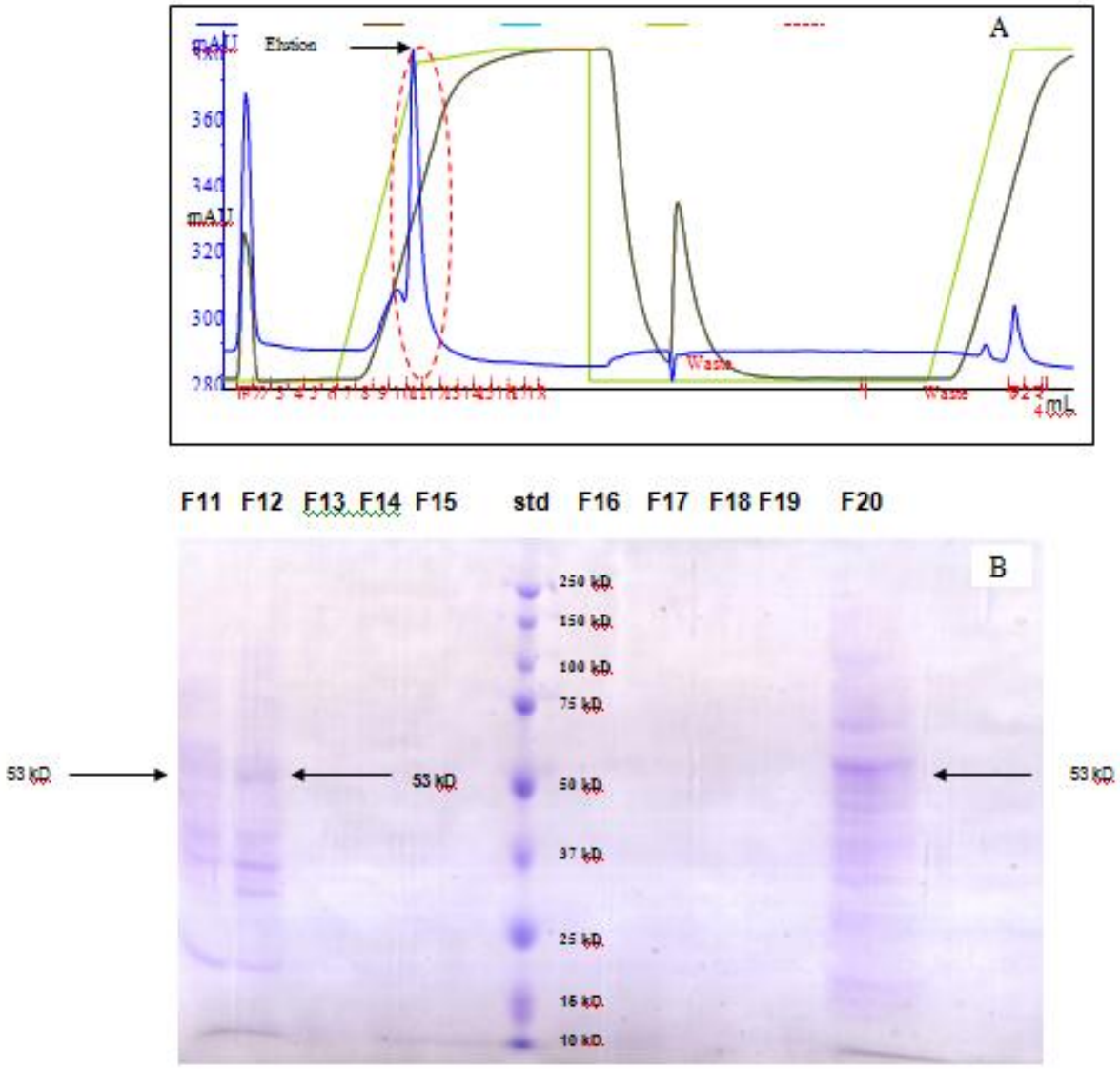

Figure 2: (A) Elution of *Rhodococcus* UKMP-5M Phenol Hydroxylase from the DEAE HITrap Fast Flow Ion-Exchange Column using 20 mMTris-HCL and 1M NaCl; Phenol Hydroxylase (Red Circle) and (B) SDS Polyacrylamide Gel of the Partially Purified *Rhodococcus* UKMP-5M Phenol Hydroxylase; Lane F11-F18, Partially Purified Fractions and Lane F20, Crude Extract

Previous report stated that phenol hydroxylase from *Rhodococcus* UPV-1 can be purified using sepharose fast-flow column, showing a dimer of 62 kD and 22 kDa (Saa *et al*., 2010). On the other hand, phenol hydroxylase from *Pseudomonas stutzeri* OX1 showed three values of molecular weight, which were 13, 38 and 60 kDa when purified using Q-sepharose fast flow, sephacryl S300 high resolution and superose

12 PC (Cafaro *et al*., 2004). Phenol hydroxylase of *Candida tropicalis* purified using DEAE-sepharose, fractionated by polyethylene glycol 6000 and gel permeation chromatography of sepharose 4B showed a molecular weight of 240 kDa and consisted of four identical subunits with a molecular weight of 60 kDa, indicating that the enzyme is a tetramer (Paca Jr *et al*., 2007). Partial purification data of phenol hydroxylase is summarized in Table 1.

Step	Volumn (mL)	Protein (mg/ml)	Total Protein (mg)	Specific activity (Unit/mg protein)	Total activity (U/mL)	Yield (%)	Purification Fold
Crude	15	570	8550	0.00528	45.2	100	1.00
DEAE - Sepharose (Ion exchange columns)	1	120	120	0.05375	6.5	14.28	10.18

Table 1: Purification table of Phenol hydroxylase by *Rhodococcus* UKMP-5M

Substantial decreased in enzyme yields was observed after partial purification using ion exchange chromatography even though the purification fold was increased.

Conclusion

The molecular weight of phenol hydroxylase produced by *Rhodococcus* UKMP-5M is 53 kDa as successfully determined by SDS polyacralamide-gel electrophoresis and purified using ion exchange chromatography.

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